Cooperative Inhibition of Bone Morphogenetic Protein Signaling by Smurf1 and Inhibitory Smads

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Smad ubiquitin regulatory factor (Smurf) 1 binds to receptor-regulated Smads for bone morphogenetic proteins (BMPs) Smad1/5 and promotes their degradation. In addition, Smurf1 associates with transforming growth factor- β type I receptor through the inhibitory Smad (I-Smad) Smad7 and induces their degradation. Herein, we examined whether Smurf1 negatively regulates BMP signaling together with the I-Smads Smad6/7. Smurf1 and Smad6 cooperatively induced secondary axes in *Xenopus* embryos. Using a BMP-responsive promoter-reporter construct in mammalian cells, we found that Smurf1 cooperated with I-Smad in inhibiting BMP signaling and that the inhibitory activity of Smurf1 was not necessarily correlated with its ability to bind to Smad1/5 directly. Smurf1 bound to BMP type I receptors via I-Smads and induced ubiquitination and degradation of these receptors. Moreover, Smurf1 associated with Smad1/5 indirectly through I-Smads and induced their ubiquitination and degradation. Smurf1 thus controls BMP signaling with and without I-Smads through multiple mechanisms.

INTRODUCTION

Bone morphogenetic proteins (BMPs), members of the transforming growth factor- β (TGF- β) superfamily, were originally identified as osteoinductive proteins in bone that induce ectopic bone and cartilage formation in vivo. However, they are now known to be multifunctional regulators of cell growth, differentiation, apoptosis, and neurogenesis and to play important roles during embryonic development (Reddi, 1994; Hogan, 1996). More than a dozen BMP isoforms have been identified, including BMP-2, BMP-4, and BMP-7 in mammals and *decapentaplegic* gene product (DPP) in *Drosophila* (Kawabata *et al.*, 1998a).

BMPs bind to type I and type II serine/threonine kinase receptors (Miyazono *et al.*, 2001). Three type I receptors and three type II receptors have been shown to bind BMPs. Of the three BMP type I receptors, activin receptor-like kinase (ALK) 3 (also termed BMP type IA receptor; BMPR-IA) and ALK6 (also termed BMPR-IB) (ten Dijke *et al.*, 1994) are structurally similar to each other and bind BMPs, e.g.,

BMP-2 and BMP-4. ALK2 binds BMP-7 and activates Smad1-dependent pathways (Macías-Silva *et al.*, 1998). Müllerian inhibiting substance, a member of the TGF- β superfamily, has been reported to associate with ALK2 and ALK6 and to transduce BMP-like signals (Gouedard *et al.*, 2000; Clarke *et al.*, 2001; Visser *et al.*, 2001).

Smad proteins play central roles in intracellular signaling by members of the TGF- β superfamily (Heldin *et al.*, 1997). Eight different Smad proteins have been identified in mammals and are classified into three subgroups, i.e., receptor-regulated Smads (R-Smads), a common-partner Smad (Co-Smad), and inhibitory Smads (I-Smads). BMP-specific R-Smads, Smads 1, 5, and 8, transiently and directly interact with activated BMPR-Is and become phosphorylated at SSXS motifs at their C termini. Smad1/5/8 then form heteromeric complexes with Co-Smad Smad4 and translocate into the nucleus where they regulate transcription of various target genes. In contrast to Smad1/5/8 and Co-Smad, I-Smads, including Smad6 and Smad7, stably bind to BMP-RIIs and compete with Smad1/5/8 for activation, resulting in inhibition of BMP signaling (Imamura *et al.*, 1997; Hanyu *et al.*, 2001). Smad6 also inhibits BMP signaling by forming a complex with Smad1 and by interfering with complex formation between Smad1 and Smad4 (Hata *et al.*, 1998).

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Figure 1. Smurf1 and Smad6 synergistically inhibit endogenous BMP signaling in *Xenopus* embryos. (A) Smad6 (500 pg) and Smurf1 (500 pg) RNAs were injected near the ventral midline of four-cell embryos. Resultant phenotypes are shown (top, secondary axis; middle panel, hyperdorsalized embryo). When 1000 pg of β -globin RNA was injected, embryos developed normally (bottom). (B) RNAs encoding β -globin (1000 pg), Smurf1 (1000 pg), Smad6 (1000 pg), or Smurf1 (250 pg) and Smad6 (250 pg) were injected near the animal pole of two-cell embryos. Animal caps were isolated from embryos at blastula stage 8 and cultured to stage 23. RNAs were extracted from pooled caps and control embryos and subjected to RT-PCR analysis. WE, whole embryo.

jected near the animal pole of two-cell embryos. Animal caps were isolated from embryos at blastula stage 8 and cultured to stage 23. RNAs were extracted from pooled caps and control embryos and subjected to RT-PCR analysis. WE, whole embryo.

Ubiquitin-dependent protein degradation plays key roles in various biological processes, including signal transduction, cell cycle progression, and transcriptional regulation (Hershko and Ciechanover, 1998). In the ubiquitin-proteasome pathway, E3 ubiquitin ligases play crucial roles in the recognition of target proteins and subsequent protein degradation. Of E3 ubiquitin ligases, the RING type and HECT type ligases have been well characterized in mammals. Smad ubiquitin regulatory factor (Smurf)1 was originally identified as a HECT type E3 ubiquitin ligase, which induces the ubiquitination and degradation of Smads 1 and 5 in a manner independent of signal (Zhu *et al.*, 1999). Smurf2, which is structurally similar to Smurf1, also targets Smad1 for degradation (Zhang *et al.*, 2001). Subsequently, Smurf2 was shown to associate with activated TGF- β -specific R-Smad Smad2 and to induce its ubiquitin-dependent degradation (Lin *et al.*, 2001). In addition, Smurf1 and Smurf2 interact with nuclear Smad7 and induce nuclear export of Smad7. The Smurfs-Smad7 complexes then associate with type I receptor for TGF- β (T β R-I, also termed ALK5) and enhance its turnover (Kavsak *et al.*, 2000; Ebisawa *et al.*, 2001). Thus, Smad7 interferes with TGF- β signaling by blockade of activation of R-Smads as well as by degradation of T β R-I together with Smurfs. These findings show that Smurfs negatively regulate TGF- β superfamily signaling by targeting their positive signaling components for ubiquitin-dependent degradation. On the other hand, Bonni *et al.* (2001) have demonstrated that Smurf2 binds to a transcriptional corepressor SnoN through activated Smad2 and thereby targets SnoN for ubiquitin-dependent degradation, suggesting that Smurf2 may positively regulate TGF- β superfamily signaling under certain conditions.

In this study, we demonstrated cooperative functions of Smurf1 and I-Smads in *Xenopus* embryos and in transfected mammalian cells. Furthermore, we showed that Smurf1-I-Smad complexes negatively regulate BMP signaling by down-regulation of activated BMP receptors as well as that of R-Smads.

MATERIALS AND METHODS

cDNA Construction

The original constructs of constitutively active forms of ALKs 2 through 6 (c.a.ALKs 2–6), Smad1, Smad5, Smad6, Smad7,

Smurf1(WT), Smurf1(CA), and ubiquitin (Ub) cDNAs were generated as described previously (Imamura *et al.*, 1997; Kawabata *et al.*, 1998b; Ebisawa *et al.*, 2001). Construction of deletion mutants of Smad1 and Smad5 lacking the PY motif [Smad1(Δ PY) and Smad5(Δ PY)] was performed by deleting amino acids 223–227 (Smad1) and 222–226 (Smad5), respectively, by a polymerase chain reaction (PCR)-based approach. Construction of deletion mutants of Smad5 lacking the MH1 domain [Smad5(Δ MH1)] was performed by deleting amino acids 1–146 by a PCR-based approach. Myc and 6Myc indicate a single copy and six tandem copies of the myc epitope tag, respectively (Kawabata *et al.*, 1998b).

Xenopus Embryo Manipulation and Microinjection

Embryo manipulations and microinjections were performed as described previously (Cho *et al.*, 1991). RNAs were injected into the animal pole at the four-cell stage or into the marginal zone of a ventral blastomere at the four-cell stage. FLAG-tagged human Smurf1 and mouse Smad6 coding sequences were subcloned into pCS2+ vector by using *Eco*RI and *Xho*I restriction sites. Capped synthetic RNA was generated by *in vitro* transcription of linearized templates by using a Megascript kit (Ambion, Austin, TX).

Reverse Transcription (RT)-PCR

RNA was isolated from pooled (at least 15) animal caps and RT-PCR analysis was performed as described previously (Nakayama *et al.*,

Table 1. Cooperative effects of Smad6 and Smurf1 on the formation of dorsalized phenotypes

Injected RNA (pg)			Dorsalized phenotypes (%)			
Smurf1	Smad6	β -Globin	Secondary axis	Hyperdorsalized	Total	n
0	0	1000	0	0	0	33
1000	0	0	26	6	32	31
500	0	0	20	0	20	35
250	0	0	0	0	0	36
0	1000	0	21	11	32	28
0	500	0	11	0	11	35
0	250	0	0	0	0	19
500	500	0	35	23	58	26
250	250	0	19	9	28	32
125	125	0	14	0	14	37
68	68	0	0	0	0	20

Figure 2. Smurf1 cooperates with Smad6 in cultured cells. (A) Effects of Smurf1 on the transcriptional activity of c.a.ALK6 in the presence of Smad5(WT) or Smad5(ΔPY) were examined using 3GC2-Lux assay. R mutant Mv1Lu cells were cotransfected with 3GC2-lux luciferase construct and various combinations of c.a.ALK6, Smad5(WT), Smad5(ΔPY), Smad7, and Smurf1 cDNAs. + and ++ are 0.1 and 0.3 μg of DNA, respectively, transfected in R mutant cells. (B) Binding of Smurf1(CA) to Smad5(WT) and Smad5(ΔPY) was examined in transfected COS7 cells. A Smurf1 mutant Smurf1(CA), which has a mutation in the HECT domain and fails to recruit ubiquitin ligase activity, was used for binding assays, including this experiment. COS7 cells were transfected with the indicated plasmids and subjected to FLAG-immunoprecipitation (IP) followed by Myc-immunoblotting (Blot). The top panel shows the interaction and the lower three panels the expression of each protein.

1998) by using the following PCR conditions: 94°C for 5 min, followed by a variable number of cycles at 94°C for 30 s; 55°C for 30 s, and 72°C for 2 min. neural cell adhesion molecule (NCAM), muscle actin, and histone H4 primers have been described previously (Nakayama *et al.*, 1998). PCR products were visualized on ethidium bromide-stained agarose gels.

Luciferase Assay

R mutant mink lung epithelial (Mv1Lu) cells were transiently transfected with various combinations of a 3GC2-lux promoter-reporter construct, expression plasmids, and pcDNA3 by using FuGENE6 (Roche Applied Science, Indianapolis, IN). Twenty-four hours after

transfection, cell lysates were prepared. Luciferase activity was measured by the dual-luciferase reporter system (Promega, Madison, WI). Total amounts of transfected DNAs were the same in each experiment, and values were normalized using *Renilla* luciferase activity.

Transfection, Immunoprecipitation, and Immunoblotting

COS7 cells or 293T cells were transiently transfected using FuGENE6. Twenty-four hours after transfection, cells were lysed with Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40). Immunoprecipitation and immunoblotting were performed as described previously (Ebisawa *et al.*, 2001). For inhibition of proteasomal degradation, cells were incubated with 2.5 μM lactacystin (Calbiochem, San Diego, CA) for 24 h, except for the experiment in Figure 5A in which cells were treated with 10 μM lactacystin for 6 h.

Pulse-Chase Analysis

COS7 cells were transiently transfected using FuGENE6. Cells were labeled for 10 min at 37°C with 50 mCi/ml [³⁵S]methionine and cysteine (Amersham Biosciences, Piscataway, NJ) in methionine- and cysteine-free DMEM, and chased in DMEM supplemented with 0.2% fetal bovine serum for the time periods indicated, as described previously (Fukuchi *et al.*, 2001). Cells were then lysed and subjected to immunoprecipitation followed by SDS-PAGE. The gels were fixed, dried, and examined using a Fuji BAS 2500 bio-imaging analyzer (Fuji Photo Film, Tokyo, Japan).

RESULTS

Smurf1 Cooperates with Smad6 to Inhibit BMP Signaling in *Xenopus* Embryos

Although both Smad6 and Smurf1 inhibit BMP signals in vivo (Tsuneizumi *et al.*, 1997; Hata *et al.*, 1998; Nakayama *et al.*, 1998; Zhang *et al.*, 2001), the functional interaction between Smad6 and Smurf1 has not been elucidated. In *Xenopus* embryos, ventral overexpression of inhibitory Smad6 suppresses BMP signaling and induces an ectopic Spemann's organizer, resulting in the development of a secondary dorsal axis (Tsuneizumi *et al.*, 1997; Hata *et al.*, 1998; Nakayama *et al.*, 1998). We therefore attempted to determine whether Smurf1 modifies the ability of Smad6 to induce secondary axes in *Xenopus* embryos by inhibition of BMP signaling.

Coinjection of RNAs encoding Smurf1 and Smad6 into ventral blastomeres of four-cell *Xenopus* embryos caused secondary dorsal axis formation and/or a hyperdorsalized phenotype in which the trunk and tail were severely reduced or lost (Figure 1A, top and middle). In contrast, embryos developed normally when RNA encoding the β-globin was injected (Figure 1A, bottom).

We then determined the effects of various doses of Smurf1 mRNA on the induction of dorsalized phenotypes when ventrally injected at the four-cell stage. Injection of as little as 500 pg of Smurf1 RNA was sufficient to cause secondary axis formation, and injection of successively higher doses of RNA up to 1000 pg led to a corresponding increase in the frequency of dorsalization (Table 1). Similar results were obtained for Smad6 RNA injection. We then determined the percentage of dorsalized phenotypes resulting from coinjection of Smurf1 RNA together with Smad6 (Table 1). Although injection of 250 pg of Smurf1 or Smad6 RNA alone was unable to induce secondary axes, coinjection of 250 pg

Figure 3. Smurf1 interacts with BMP type I receptors via I-Smads. Binding of Smurf1 to different type I receptors for BMPs in the presence of Smad6 (A) or Smad7 (B) was examined. COS7 cells were transfected with FLAG-tagged constitutively active forms of type I receptors for the TGF- β superfamily proteins (c.a.ALK2-FLAG, c.a.ALK3-FLAG, and c.a.ALK6-FLAG for BMPs; and c.a.ALK5-FLAG and c.a.ALK4-FLAG for TGF- β and activin) and 6Myc-tagged Smurf1(CA) in the presence or absence of 6Myc-tagged Smad6 (A) or 6Myc-tagged Smad7 (B). Cell lysates were subjected to FLAG-immunoprecipitation (IP) followed by Myc-immunoblotting (Blot). The top panels show the interaction and the lower two panels the expression of each protein.

of Smurf1 RNA with 250 pg of Smad6 RNA induced secondary axes. Moreover, when the total amount of RNA injected was decreased to 250 pg (125 pg of Smurf1 RNA and 125 pg of Smad6 RNA), we observed dorsalized phenotypes to an extent similar to that obtained by 500 pg of Smurf1 or Smad6 RNA.

To determine whether this cooperation between Smurf1 and Smad6 is due to inhibition of endogenous BMP signaling, we examined whether they induce expression of a neural-specific marker gene in ectodermal explants (animal caps). Smurf1 and/or Smad6, but not β -globin, induced expression of the pan-neural marker NCAM in animal caps, but not that of the mesodermal marker muscle actin (Figure 1B). These results suggest that Smurf1 enhances the ability of Smad6 to inhibit BMP signals in vivo.

Smurf1 Cooperates with I-Smads to Inhibit BMP Signaling in Mammalian Cells

To determine the cooperation of Smurf1 with I-Smads in mammalian cells, we next examined the effect of Smurf1 on the inhibitory activity of Smad7 in cultured cells by using a

BMP-responsive promoter-reporter construct, 3GC2-Lux (Ishida *et al.*, 2000). In transfected cells, c.a.ALK6 induced transcription from 3GC2-Lux, which was enhanced by Smad5. Smurf1 inhibited BMP signaling and Smad7 enhanced this inhibitory activity of Smurf1 (Figure 2A). Smurf1 may prevent BMP signaling independent of I-Smads, because Smurf1 interacts with Smads 1 and 5 through the PY motif and induces their ubiquitin-dependent degradation (Zhu *et al.*, 1999). We therefore generated deletion mutants of Smad5 [Smad5(Δ PY)] that lack the PY motif in the linker region and examined the effect of Smurf1 on transcriptional activity induced by Smad5(Δ PY) by using 3GC2-Lux. In contrast to wild-type (WT) Smad5, Smad5(Δ PY) failed to interact with Smurf1 (Figure 2B). As shown in Figure 2A, Smad5(Δ PY) enhanced transcriptional activity together with c.a.ALK6, similar to wild-type Smad5. Interestingly, Smurf1 suppressed transcriptional activity induced by Smad5(Δ PY), which was more prominent in the presence than in the absence of Smad7 (Figure 2A). Similar results were obtained using Smad1(Δ PY) (our unpublished data). Smurf1 thus inhibits BMP signaling not only by direct

A

c.a.ALK6-HA	-	+			
FLAG-Ub	-	+			
6Myc-Smurf1	-	WT		CA	
Myc-Smad6	-	+	-	+	+

IP: anti-FLAG
Blot: anti-HA [FLAG-Ub]_n-
c.a.ALK6-HAIP: (-)
Blot: anti-Myc ← 6Myc-Smurf1
← Myc-Smad6IP: (-)
Blot: anti-HA

← c.a.ALK6-HA

B

cDNA	c.a.ALK6-FLAG	-	+					
	FLAG-Smurf1	-	-	-	-	+	+	+
	Myc-Smad7	-	-	-	-	+	+	+
Chase time (h)		0	0	2.5	5	0	2.5	5

IP: anti-FLAG

← FLAG-Smurf1
← c.a.ALK6-FLAG

Figure 4. Smurf1-I-Smad complexes target ALK6 for ubiquitin-dependent degradation. (A) Smad6 enhances ubiquitination and degradation of ALK6 by Smurf1. Ubiquitination of c.a.ALK6 by Smad6-Smurf1 complexes was examined. 293T cells were transfected with the indicated plasmids and treated with 2.5 μ M of lactacystin for 24 h before cell lysis. Lysates from cells were subjected to anti-FLAG immunoprecipitation followed by anti-HA immunoblotting. Polyubiquitination species of constitutively active forms of ALK6 ([FLAG-Ub]_n-c.a.ALK6-HA) are indicated in the top panel. (B and C) Smurf1-Smad7 complex induced rapid turnover of ALK6. COS7 cells were transfected with c.a.ALK6-FLAG, FLAG-Smurf1, and/or Myc-Smad7. [³⁵S]methionine- and cysteine-labeled cell lysates were immunoprecipitated by FLAG antibody. Immune complexes were subjected to SDS-PAGE and examined using a Fuji BAS 2500 bio-imaging analyzer. Arrowheads indicate the premature form of ALK6.

C

cDNA	c.a.ALK6-FLAG	-	+							
	FLAG-Smurf1	-	-				+			
	6Myc-Smad7	-	-		+		-		+	
Chase time (h)		0	0	2	0	2	0	2	0	2

IP: anti-FLAG

← FLAG-Smurf1
← c.a.ALK6-FLAG

binding to R-Smads but also by additional mechanisms involving I-Smads.

Smurf1 Interacts with BMPR-Is via I-Smads

To determine the target of Smurf1–I-Smad complex in BMP signaling, we first examined whether I-Smads act as adapter molecules that link BMPR-Is to the ubiquitin-proteasome pathway. In transfected COS7 cells, Smad6 enhanced the interaction of Smurf1 with constitutively active forms of activin type I receptor (c.a.ALK4) and T β R-I (c.a.ALK5), as well as with those of BMPR-Is (c.a.ALK2, c.a.ALK3, and c.a.ALK6) (Figure 3A). Similar results were obtained using Smad7 instead of Smad6 (Figure 3B). These findings suggest that Smurf1 is recruited to BMPR-Is through Smads 6 and 7.

Smurf1–I-Smad Complexes Induce Ubiquitination and Degradation of BMPR-Is

To determine whether Smurf1–I-Smad complexes act as E3 ubiquitin ligase complexes for BMPR-Is, ubiquitination of c.a.ALK6 by Smurf1–I-Smad complex was investigated in mammalian cells. Although Smurf1 alone ubiquitinated c.a.ALK6 weakly, Smads 6 and 7 enhanced receptor-ubiquitination by Smurf1 (Figure 4A; our unpublished data). Next, to investigate whether Smurf1–I-Smad complexes regulate degradation of BMPR-Is, we examined the effects of Smad7 and Smurf1 on turnover of ALK6 in pulse-chase experiments. As shown in Figure 4, B and C, c.a.ALK6 proteins were observed as two types of differentially migrating bands. Because membrane receptors are posttranslationally modified by addition of N-linked oligosaccharides, the rapidly migrating bands at time 0 may represent a premature form of ALK6 (Figure 4, B and C, arrowhead), whereas the slowly migrating bands may represent its mature form. Smurf1 and Smad7 strongly induced the degradation of c.a.ALK6 (Figure 4B). Notably, c.a.ALK6 was more efficiently degraded in the presence of Smurf1 and Smad7 than in the presence of either Smurf1 or Smad7 alone. These results suggested that ubiquitin-dependent degradation of ALK6 is cooperatively mediated by Smurf1 and Smad7.

Smad6 Recruits Smurf1 into a Complex with Activated BMP-specific R-Smads

Although Smad6 has been reported to bind to BMP receptors and inhibit activation of Smad1/5, Hata *et al.* (1998) demonstrated that Smad6 also binds to activated Smad1 and inhibits complex formation between Smad1 and Smad4. We therefore investigated whether Smurf1 can associate with Smad1/5 through Smad6. We first tested the effect of proteasomal inhibitor on the interaction between Smad1 and Smad6. As shown in Figure 5A, only very weak interaction of Smad1 with Smad6 was observed in the absence of the proteasomal inhibitor lactacystin. In contrast, association of Smad1 with Smad6 was enhanced in the presence of lactacystin, suggesting that the Smad1-Smad6 complex is degraded by proteasomes. To elucidate whether Smurf1 is linked to this degradation, we next examined the effect of Smad6 on the binding of Smurf1 to Smad5. Although Smurf1 binds to Smad5 directly, Smad6 enhanced interaction between Smad5 and Smurf1 (Figure 5B). These findings suggest that Smurf1 binds to BMP-specific R-Smads not only directly but also indirectly through Smad6.

Figure 5. Smad6 recruits BMP-specific R-Smads to Smurf1 and enhances their ubiquitination and degradation. (A) Association of Smad6 with Smad1 is enhanced by treatment with proteasomal inhibitor lactacystin. COS7 cells were transfected with the indicated plasmids and treated with 2.5 μ M lactacystin for 24 h before cell lysis. Lysates from cells were subjected to FLAG-immunoprecipitation (IP) followed by Myc-immunoblotting (Blot). (B) Interaction of Smurf1 with Smad5 is enhanced in the presence of Smad6. COS7 cells were transfected with the indicated plasmids, and lysates from cells were subjected to FLAG-IP followed by Blot.

Smurf1–I-Smad Complexes Induce Ubiquitination and Degradation of BMP-specific R-Smads

To determine whether Smurf1–I-Smad complexes act as E3 ubiquitin ligase complexes for BMP-specific R-Smads, ubiquitination of Smads 1 and 5 by Smurf1–I-Smad complexes was examined in transfected 293T cells. Polyubiquitinated Smad1 was immunoprecipitated from cell lysates by using anti-FLAG antibody to isolate FLAG-tagged Smad1, followed by immunoblotting with anti-hemagglutinin (HA) antibody to detect polyubiquitin on target proteins (Figure 6A). As shown in Figure 6A, top, Smads 6 and 7 induced ubiquitination of Smad1 by Smurf1(WT). Smurf1(CA) could not induce ubiquitination of Smad1 even in the presence of Smad6/7. In the next experiment, cell lysates were subjected to HA-tagged ubiquitin immunoprecipitation, followed by Myc-tagged Smad1 immunoblotting. Consistent with the result shown in Figure 6A, Smad6 enhanced ubiquitination of Smad1 by Smurf1 (Figure 6B). Moreover, in the absence of Smad6, Smurf1 did not ubiquitinate Smad1(Δ PY) but could do so in the presence of Smad6 (Figure 6B, lanes 8 and 9 from the left).

Figure 6. Smad6/7 enhances ubiquitination and degradation of Smad1/5 by Smurf1. Ubiquitination of Smad1 (A and B) or Smad5 (C) by Smurf1 was examined. 293T cells were transfected with the indicated plasmids and treated with 2.5 μ M lactacystin for 24 h before cell lysis. Lysates from cells were subjected to anti-FLAG immunoprecipitation followed by anti-HA immunoblotting (A) or anti-HA immunoprecipitation followed by anti-Myc immunoblotting (B and C). Polyubiquitinated species of Smads, $[HA-Ub]_n$ -FLAG-Smad1 (A), $[HA-Ub]_n$ -6Myc-Smad1 (B), and $[HA-Ub]_n$ -6Myc-Smad5 (C), are indicated in the top panels. Levels of expression of Smad1 or 5, Smad6/7, and Smurf1 were confirmed by immunoblotting and are shown in the bottom two panels.

It is important to determine whether interaction of Smad1/5 with the Smurf1-Smad6 complex and their ubiquitination by Smurf1-Smad6 occur ligand dependently. We therefore generated a deletion mutant of Smad1 [Smad1(Δ MH1)], which lacks the N-terminal Mad homology 1 (MH1) domain to mimic activated Smad1, and examined its ubiquitination by the Smurf1-Smad6 complex. As shown in Figure 6C, Smurf1 induced ubiquitination of Smad1(Δ MH1) more strongly than it did that of full-length Smad1 [Smad1(Full)], suggesting that the Smurf1-Smad6 complex targets activated R-Smads more efficiently than nonactivated R-Smads.

DISCUSSION

Previous studies showed that both Smad6 and Smurf1 inhibit BMP signals *in vivo* (Tsuneizumi *et al.*, 1997; Hata *et al.*, 1998; Nakayama *et al.*, 1998; Zhang *et al.*, 2001). However, the functional interaction between Smad6 and Smurf1 has not been elucidated. In this study, we have demonstrated that Smurf1 cooperates with I-Smads to inhibit BMP signaling in *Xenopus* embryos. Moreover, in mammalian cells, we showed that

Smurf1 inhibits BMP signaling together with I-Smads and that Smurf1 can inhibit BMP signaling not by direct binding to R-Smads. These results suggest that Smurf1 cooperates with I-Smads to inhibit BMP signaling.

Smurf1 was originally identified as an E3 ubiquitin ligase that specifically induces the ubiquitination and degradation of BMP-specific R-Smads Smads 1 and 5 in a signal-independent manner (Zhu *et al.*, 1999). Thus, Smurf1 was suggested to determine the competence of cells to respond to BMP signaling by controlling cytoplasmic pools of R-Smads. Smurf2 is structurally highly related to Smurf1 and induces ubiquitin-dependent degradation of Smad1 as well as that of a TGF- β -specific R-Smad, Smad2 (Lin *et al.*, 2001; Zhang *et al.*, 2001). In addition, Smurf1 and Smurf2 physically interact with I-Smads Smad6 and Smad7. Smurfs induce nuclear export of Smad7, associate with T β R-I, and enhance its turnover (Kavsak *et al.*, 2000; Ebisawa *et al.*, 2001). Thus, Smurfs have been shown to down-regulate BMP signaling by targeting R-Smads for ubiquitin-dependent degradation and to inhibit TGF- β signaling by inducing receptor degradation together with I-Smads. In the present study, we

Figure 7. Inhibition of BMP signaling by Smurf1 through multiple mechanisms. In the absence of BMP signaling, Smurf1 interacts with BMP-specific R-Smads and induces their degradation to maintain low basal levels of them. In the presence of BMP signaling, Smurf1 targets activated type I receptors and BMP-specific R-Smads for ubiquitin-dependent degradation. I-Smads, induced by BMPs, act as adaptors in this process.

showed that Smurf1 inhibits BMP signaling by degradation of BMP type I receptors as well as that of R-Smads through I-Smads.

Smad pathways are conserved in vertebrates and in *Drosophila*. DSmurf1 was recently identified as a *Drosophila* ortholog of Smurfs 1 and 2 (Podos *et al.*, 2001). The function of DSmurf1 is restricted to the BMP-2/4 ortholog DPP pathway during development, suggesting important roles for Smurfs in BMP signaling in *Drosophila*. In this case, DSmurf1 seems to control amounts of the Smad1/5 ortholog Mothers against dpp and to down-regulate accumulation of activated Mothers against dpp by receptor turnover, which is in agreement with the findings observed in the present study. It will be interesting to determine whether Daughters against dpp, a *Drosophila* I-Smad, and DSmurf1 functionally synergize in vivo.

Because Smurf1 enhances the ability of I-Smads to inhibit BMP signaling both in *Xenopus* embryos and in cultured cells, we studied the molecular mechanisms that govern these cooperative effects. We showed that Smurf1 interacted with activated BMPRI-S through Smad6/7 and induced their ubiquitin-dependent degradation, similar to the effects of Smurfs-Smad7 complexes on TGF- β signaling. In addition to binding to receptors, Smad6 has also been reported to bind to activated Smad1 (Hata *et al.*, 1998). Interestingly, we found that although Smurf1 bound to Smad1/5 directly, Smad6/7 enhanced binding of Smurf1 to Smad1/5 and ubiquitination of Smad1/5 by Smurf1. These findings are particularly important for another BMP-specific R-Smad,

Smad8. In contrast to Smads 1 and 5, Smad8 lacks the PY motif in its linker region. However, the present findings suggest that Smad8 may also be degraded by Smurf1 in the presence of I-Smads.

An important question is whether interaction of Smad1/5 with the Smurf1-Smad6 complex and their ubiquitination by Smurf1-Smad6 occur in a ligand-dependent manner. It was previously reported that expression of I-Smads is induced by BMPs (Nakao *et al.*, 1997; Takase *et al.*, 1998) and that Smad1 binds to Smad6 in a ligand-dependent manner (Hata *et al.*, 1998). Thus, it is likely that interaction of Smad1/5 with Smurf1 through I-Smads depends on BMP signaling in mammalian cells. However, because overexpression of I-Smads inhibits activation of Smads 1 and 5 by BMP receptors, we were not able to demonstrate the effect of I-Smads on the link of Smurf1 to activated Smad1/5 (our unpublished observation). We therefore used Smad1(Δ MH1), lacking the N-terminal MH1 domain to mimic activated Smad1, and found that Smurf1 induced ubiquitination of Smad1(Δ MH1) more efficiently than Smad1(Full). These findings suggest that the Smurf1-Smad6 complex targets activated R-Smads more efficiently than nonactivated R-Smads.

In conclusion, we demonstrated cooperative inhibition of BMP signaling by Smurf1 and I-Smads. We propose inhibition of BMP signaling by Smurf1 through multiple mechanisms (Figure 7). Smurf1 has been reported to degrade Smad1/5 in a ligand-independent manner (Zhu *et*

al., 1999). Thus, Smurf1 regulates the amplitude of the cellular response to BMPs by limiting pools of BMP-specific R-Smads in the cytoplasm. Moreover, we demonstrated in the present study that Smurf1 acts as an E3 ubiquitin ligase together with I-Smads to control the intracellular signaling of BMPs. There are two possible mechanisms of action of the Smurf1-I-Smad complexes. They interact with activated BMPR-Is to prevent activation of BMP-specific R-Smads and remove the receptors by ubiquitin-dependent degradation. In addition, Smurf1 binds to BMP-specific R-Smads through I-Smads and induces their ubiquitin-dependent degradation. Smurf1 and I-Smads thus reset the Smad pathway for interpretation of subsequent BMP signaling.

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